PECTIC POLYSACCHARIDES IN COTYLEDONS OF KIDNEY BEANS

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The pectic substances of plant tissues comprise a group of polysaccharides containing galacturonic acid, arabinose and galactose as the main sugar residues. There are differences among pectic substances in the proportion of uronic acid to neutral sugar units. Stoddart et al. (1) reported that at least three distinct fractions, a neutral arabinan-galactan, a weakly acidic complex pectinic acid and a mixture of more acidic pectinic acids of varying degrees of methyl esterification, were present in the pectins of sycamore callus and sycamore cambium tissues which were extracted with a sodium hexametaphosphate solution brought to pH 3.5 to 4.0 with hydrochloric acid.

Thermal maceration of peas and beans, like that of many other plant tissues, seems to be caused by the dissolution of intercellular pectic substances. Furthermore, a complex reaction system involving pectic substances is considered to be responsible for the occurrence of "hard-seededness"(2). The information available concerning the components of pectins prepared from cotyledons of legumes and their chemical structures is somewhat limited. Sasaki and Yamashita(3) prepared pectin-like polysaccharides by extracting powdered embryos of soybeans with 0.5% ammonium oxalate or water at 90°C after treatment with cold 0.2% NaOH. The preparation had a much lower uronic acid content than fruit pectins(4) and did not precipitate with $CuSO_4$ and Fehring's solutions. Kikuchi(5) isolated from the hot-water-extract of soybeans an arabinogalactan, a galacturonan-arabinogalactan and a pectinic acid containing 71% of anhydrogalacturonic acid.

In the present study pectic polysaccharides were extracted from the cotyledons of kidney beans under conditions designed to avoid their degradation and separated into several fractions by DEAE-cellulose chromatography. Sugar composition and enzymatic susceptibility of these fractions were investigated, with the aim of elucidating the chemical structures of pectic polysaccharides of legume cotyledons. The results of these investigations are described below.

MATERIALS AND METHODS

Determination of total sugars and uronic acid. The methods for

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determination of total sugars and uronic acid were the same as described in a previous paper(6).

Paper chromatography. The sample to be analyzed was heated in 1 N H_2SO_4 for 60 min. at 120°C and to the hydrolyzate was added $BaCO_3$ to remove SO_4^{2-}. After centrifugation the hydrolyzate was freed from Ba^{2+} on an ion exchanger resin and placed on Toyo No. 52 filter paper. Ascending and two-dimensional chromatography was carried out with n-butanol . pyridine . water (6 : 4 : 3) and phenol . water (5 : 1) as solvent systems.

Extraction of pectic polysacccharides. After kidney beans (*Phaseolus vulgaris,* "Uzuramame"; whole seeds) had been soaked overnight at 5°C, their seed coats and hypocotyls were removed and discarded. The cotyledons obtained (total solids, 48.9%) were cut widthwise into slices 0.5 to 1 mm thick. The slices (300g) were weighed into 1-*l* flask and extracted with 500 m*l* of 0.035 M ammonium oxalate-oxalic acid, pH 4.25, in a water bath (75°C) for 8 hr. During extraction 0.035 M oxalic acid was added to the flask to keep the pH of the extractant at 4.25. After cooling under running water the liquid was filtered through a sintered glass filter (G-4). The residue was returned to the flask and re-extracted with 500 m*l* of 0.035 M ammonium oxalate-oxalic acid, pH 4.25. This process was repeated (ten times in all) until the extracts gave only a feeble carbazole reaction for uronic acid.

Determination of degree of esterification. This was made by a slight modification of Hirota's method(7). For the qualitative analysis the sample, after deesterification with alkali and neutralization with dilute sulfuric acid, was dialyzed against deionized water. The dialyzate was analyzed for methanol by the chromototropic acid method.(8)

Polygalacturonases. Carrot exopolygalacturonase (CPG) and the endopolygalacturonase of *Saccharomyces fragilis* (YPG) were prepared as described in previous papers (9, 10).

Estimation of limit value of enzymatic degradation. The reaction mixture contained 1 to 1.5 mg of pectic polysaccharides, 1.5 units of CPG, 7.5 units of YPG and 50μ moles of acetate buffer, pH 4.65, in a total volume of 1 m*l*. Incubation was for 5 days at 27°C(9, 11). The release of aldehyde groups was estimated by the Hatanaka modification(12) of the Willstätter-Schudel method.

RESULTS

1. *DEAE-Cellulose Chromatography of the Extract from Kidney Bean Cotyledons.*

By DEAE-cellulose chromatography, five fractions were obtained from the extracts from kidney bean cotyledons described in Methods

(Table 1). Fraction I corresponded to neutral pectic polysaccharide,

TABLE 1. Fractionation of pectic polysaccharides of kindey bean cotyledons by DEAE-cellulose chromatography

Eluting solution	Acetate buffer				NaOH
	0.02 M (washings)	0.1 M	0.2 M	0.3~0.8 M (linear gradient)	0.1 N
Fraction	I	II	III	IV	V
Percentage of total pectic polysaccharides	55.7	6.8	20.1	16.0	1.4

The extracts from kidney bean cotyledons described in Methods, after being pooled and clarified by centrifugation, were concentrated under reduced pressure and dialyzed in a cellulose tube (Visking, 0.001 inch thick) against deionized water. Concentration and dialysis were repeated alternatively and the last concentrate was dialyzed against 0.02 M acetate buffer, pH 6.0 (pectic polysaccharides in the dialyzed solution, 19.7g; loss of the polysaccharides by dialysis, 2.5%). Part of the dialyzed solution was added to a DEAE-cellulose column (3.4×14 cm) equilibrated with 0.02 M acetate buffer, pH 6.0, and the column was washed with the same buffer. Elution was carried out successively with 0.1, 0.2 and 0.3~0.8 (linear gradient) M acetate buffer, pH 6.0, and finally 0.1 N NaOH. Each eluting solution was changed to the next (or elution was stopped) when the carbazole reaction of the eluates became feeble. Tubes were combined in five fractions (I~V).

exceeding the others in quantity. Fractions II and IV were rechromatographed, the elution curves being shown in Fig. 1. Table 2 shows the

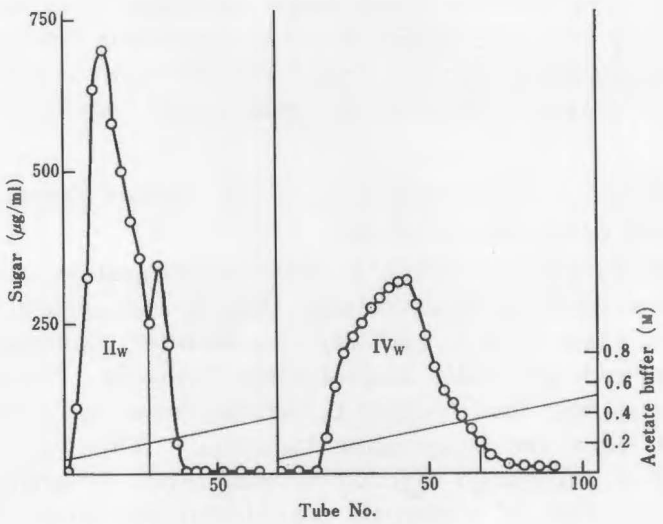

Fig. 1. DEAE-Cellulose chromatorgraphy of fractions II and IV (Table 1). Fractions II and IV were added to each DEAE-cellulose column (3.4×10 cm) equilibrated with 0.02 M acetate buffer, pH 6.0. After being washed with the same buffer, the column was eluted with a linear gradient (0.1~0.8 M) of acetate buffer, pH. 6.0.

TABLE 2. Uronic acid content, esterification degree and enzymatic susceptibility of pectic polysaccharides in fractions II_w, IV_w (Fig. 1) and V (Table 1)

Fraction	Uronic acid (%)	Degree of esterification (%)	Galacturonic acid produced by CPG+YPG*	Enzymatic degradation limit* (%)
II_w	12.5	17.4	−?	0
IV_w	31.2	43.7	+	0.8
V	27.0	−	++	20.8

Galacturonic acid was detected by paper chromatography. Enzymatic degradation limits were estimated by the same method as described in a previous paper[9].

*The composition of reaction mixture and the conditions of incubation for the detection of galacturonic acid were the same as those for the estimation of enzymatic degradation limits. Amounts of galacturonic acid produced were indicated by ++ (medium), +(small) and −(no). No other degradation products than galacturonic acid were observed on paper chromatograms.

results of an experiment on the properties of pectic polysaccharides in fractions II_w and IV_w (Fig. 1.) and fraction V (Table 1). As the pectins in all of the fractions have a low uronic acid content, they are considered to belong to weakly acidic pectic polysaccharide. Fraction II_w was hardly degraded by CPG plus YPG. In contrast, galacturonic acid was produced in an appreciable amount from fraction IV_w or V, though its amount was far smaller than that from commercial pectic acids. The percentages of esterification of fractions II_w and IV_w were 17.4 and 43.7, respectively. The fact that these values are higher than the percentage of limited enzymatic degradation of the corresponding fractions indicates that polygalacturonase-resistant residues in the molecules of the weakly acidic pectic polysaccharide are at least partly esterified by methyl groups.

2. *DEAE-Cellulose Chromatography of the Extract from Kidney Bean Cotyledons after Deestrification.*

The dialyzed solution (Table 1), after deesterification, was chromatographed on a DEAE-cellulose column (Fig. 2) and separated into four fractions (fractions A, B, C, and D). These were examined for uronic acid content and enzymatic susceptibility (Table 3). Fraction A was eluted more rapidly than fraction B, but the former had a higher uronic acid content than the latter, notwithstanding. This was true of fractions C and D. Although fraction C was richer in uronic acid than fraction V, the limit of enzymatic degradation was lower than that of the latter. Table 4 shows the neutral sugar composition of fractions I, V (Table 1), A, B, and C (Table 3). It seems likely that, in the neutral polysaccharide of fraction I and weakly acidic pectic polysaccharides of fractions V, A, B, and C, xylose is present in larger amounts than galactose.

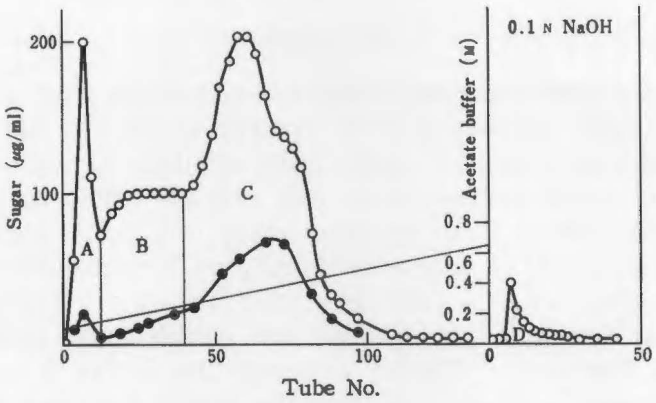

Fig. 2. DEAE-Cellulose chromatography of the deesterified pectic polysaccharides of kidney bean cotyledons. Another part of the dialyzed solution that was chromatographed in the experiment of Table 1, after alkali-treatment, neutralization, dialysis, and concentration, was added to a DEAE-cellulose column (3.4×10 cm) equilibrated with 0.02 M acetate buffer, pH 6.0, and the column was washed with the same buffer. Pectic polysaccharides were eluted with a linear gradient (0.1→0.8 M) of acetate buffer, pH 6.0, and finally 0.1 N NaOH. ○——○ Total sugar, ●——● uronic acid, —— acetate buffer.

TABLE 3. Uronic acid content and enzymatic susceptibility of pectic polysaccharides in fractions A, B, C and D (Fig. 2)

Fraction	Uronic acid (%)	Galacturonic acid produced by CPG+YPG*	Enzymatic degradation limit (%)
A	14.1	−?	0.4
B	12.6	−?	0
C	35.3	+	5.0
D	28.5	++	−

*Amounts of galacturonic acid produced were indicated by ++(medium), +(small) and −(no). No other degradation products than galacturonic acid were observed on paper chromatograms.

TABLE 4. Neutral sugar composition of pectic polysaccharides in fractions I, V (Table 1), A, and C (Table 3).

Fraction	Arabinose	Xylose	Rhamnose	Fucose	Galactose	Glucose
I	++	++	−	+	+	±
V	++	+	±	+	+	−
A	++	+	−	+	+	−
B	++	+	−	+	+	−
C	++	++	±	+	+	−
D	++	+	±	+	+	−

Amounts of sugars were indicated by ++(large), +(small), ±(trace) and −(no).

DISCUSSION

The pectic polysaccharides, which are extractable from plant tissues with the reagents complexing with divalent metal ions can be divided into three groups: neutral pectic polysaccharide (arabinan-galactan), weakly acidic pectic polysaccharide and strongly acidic pectic polysaccharide. The ratio of these polysaccharides in a pectin seems to vary according to the state of development and growth conditions of the tissue from which the pectin is prepared rather than the individual species. Stoddart et al. assumed that strongly and weakly acidic pectic polysaccharides are fanctionally distinct and that the former is more characteristic of division and cell-plate formation, rather than that of extension, whereas the latter could be involved in the process of extension. A common polysaccharide precursor of both strongly and weakly acidic pectic polysaccharides is possibly the neutral polysaccharide serving as the neutral block donner.

In a previous study(13) DEAE-cellulose chromatography with acetate buffer and 0.1 N NaOH as eluting agents was successfully applied for the separation of pectic substances. In the present study pectic polysaccharides of kidney bean cotyledons also were fractionated by this chromatographic method. Of the weakly acidic pectic polysaccharide fractions obtained, some were hardly degraded by polygalacturonases, but the others were susceptible to the enzymes though the values of limits of enzymatic degradation were lower than those of commercial pectic acids. It is generally accepted that, in the molecules of weakly acidic pectic polysaccharide, neutral sugar blocks are present attached to, or interspersed in, the polyuronide chains. The above finding indicates that weakly acidic pectic polysaccharide is built up of one or more polygalacturonic acid chains, which may be absent in some cases, and of one or more polygalacturonase-resistant sugar blocks, which contain galacturonic acid residues together with neutral sugar blocks. The content of polygalacturonic acid chains in the weakly acidic pectic polysaccharide of kidney bean cotyledons was only 5.6% (20.8×0.27) even in the highest case. It is well known that the extent of hydrolysis of pectinic acid by endopolygalaturonase is roughly in inverse proportion to that of esterification. This means that polygalacturonase-susceptible galacturonic acid residues in pectinic acid are partly or wholly esterified by methyl groups. Stoddart, et al. reported that carboxyl groups of sycamore callus and sycamore cambium pectins were unesterified. The present study, however, showed that the weakly acidic pectic polysaccharide of kidney bean cotyledons contains methyl ester groups. The fact that the percentage of esterification of the weakly acidic pectic polysaccharide is higher than that of the limited enzymatic degradation

indicates that polygalacturonase-resistant galacturonic acid residues of the pectic polysaccharide are at least partly esterified by methyl groups.

Pectic polysaccharides containing methyl ester groups are degraded by a transelimination mechanism in warm neutral solutions(14, 15) or in alkaline solutions(16) at room temperature. In this study care was taken not to subject pectic polysaccharide solutions to these conditions. The pectin of kidney bean cotyledons prepared with such care contained the neutral polysaccharide and weakly acidic pectic polysaccharide components. Apple-fruit pectin was classified by Stoddart et al. as a weakly acidic pectic polysaccharide according to its electrophoretic mobility. It is reasonable, however, to consider apple-fruit pectin as belonging to strongly acidic pectic polysaccharide because its uronic acid content is as high as 87%(4). Strongly acidic pectic polysaccharide, the main component of cambium and callus tissue pectins, is probably absent from the pectin of kidney bean cotyledons. In contrast, the neutral polysaccharide component was present in extraordinarily large amount(1, 4). Rees and Wight(17) reported that the proportion of 1-4 linked α-galacturonic acid residues to the other sugar ones was low in the whole pectin of white mustard cotyledons. It seems desirable to make wider studies on the components of pectic polysaccharide of cotyledons and other plant tissues.

The weakly acidic pectic polysaccharide of kidney bean cotyledons has a considerably high methyl ester content. For this cause it can break down by the transelimination mechanism in hot solution. The degree of esterification of the pectic polysaccharides in cell walls is considered to be one of the important factors infiuencing thermal maceration of not only vegetables but also legumes in cooking.

SUMMARY

Pectic polysaccharides were extracted from cotyledons of kidney beans under conditions designed to avoid their degradation and separated into a fraction of neutral polysaccharide and those of weakly acidic pectic polysccaharides containing 12.5 to 35.3% of galacturonic acid. Some of the latter fractions could hardly be degraded by polygalacturonases, while the rest of the latter were susceptible to the same enzymes, though the values of degradation limits were considerably lower than those of commercial pectic acids. It was found that polygalacturonase-resistant galacturonosyl residues in the molecules of the weakly acidic pectic polysaccharides are at least partly esterified by methyl groups. Strongly acidic pectic polysaccharide, the main component of cambium and callus pectins, is probably absent from the pectin of kidney bean cotyledons. In contrast, the neutral polysaccharide component is present in extraordinarily large amount.

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